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Familial and Metachronous Malignant Lymphoma: Absence of Constitutional p53 Mutations

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Familial and metachronous aggregations of malignant lymphoma are well-documented, but the molecular basis of a predisposition for development of lymphoma is as yet unclear. Malignant lymphomas have been described as part of the spectrum of neoplasias in Li-Fraumeni syndrome (LFS), which is associated with constitutional mutations of p53. However, p53 germline mutations have also, albeit less frequently, been described in patients not fitting the clinical definition of LFS. To clarify whether a genetic predisposition for lymphoma is associated with constitutional p53 mutations, DNA from normal blood lymphocytes of 12 lymphoma patients with a family history of lymphoma and/or with metachronous lymphoma (median age 37 years) was examined for mutations of p53 exons 4–8. One patient had four first-degree relatives with Hodgkin's disease, acute leukemia, and carcinomas, but the family history did not fulfill criteria of LFS. Four patients with Hodgkin's disease were diagnosed with metachronous non-Hodgkin's lymphoma as a second malignant neoplasm. No constitutional p53 mutations were detected in any of these patients, implying that outside the clinical spectrum of LFS, constitutional p53 mutations are rare in patients with lymphomas. Am. J. Hematol. 62:144–149, 1999.

Key words: hereditary cancer; Hodgkin's disease; Li-Fraumeni syndrome; non-Hodgkin's lymphoma; tumor suppressor genes

INTRODUCTION

Familial aggregation of malignant lymphoma is an infrequent, albeit well-documented observation [1,2]. The incidence of Hodgkin's disease (HD) has been calculated to be elevated 3–9 fold in relatives of patients with this disease [1], and monozygotic twins have an even higher concordance of HD relative to dizygotic twins [3]. Therefore, a genetic basis of predisposition to HD (4–7) and non-Hodgkin's lymphoma (NHL) [4] has been discussed. Similarly, patients with HD or NHL (with or without a family history of lymphoma) have a variably increased risk of developing metachronous lymphoma. Although radiochemotherapy has been defined as a risk factor for subsequent malignancies, a genetic predisposition for development of metachronous lymphoma had been proposed ([8] and references therein). Thus far, however, no constitutional monogenic alterations have been identified in patients with familial aggregation of lymphoma.

Heterozygous, constitutional p53 mutations predispose affected individuals to a wide spectrum of malignancies including soft tissue sarcomas, osteosarcomas, leukemias, brain tumors, adrenal carcinomas, breast cancer, and less frequently, other neoplasias including lymphoma, often at an early age [9,10]. This rare familial syndrome of tumor predisposition, first described by Li and Fraumeni [11], is defined by a proband with sarcoma before 45 years of age, a first-degree relative with cancer in this age group and another first- or second-degree relative with either cancer up to this age or sarcoma at any age [12]. Afflicted individuals have an increased rate of secondary malignancies following radiochemotherapy of their primary malignancy [13]. Germ-

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line p53 mutations are less frequently found in cancer families which do not fit the rigorous definition of LFS, especially in breast cancers and sarcomas, and in patients with multiple primary cancers, including NHL [14–16].

Hematologic neoplasms only infrequently exhibit somatic p53 mutations [17]. Notable exceptions are Burkitt's lymphoma [18], AIDS-associated NHL [19], and HTLV-I-associated acute T-cell leukemia/lymphoma [20]. Reed-Sternberg cells have also been shown to harbor p53 mutations [21–23]. Further evidence for a possible role of p53 inactivation in lymphomagenesis stems from a model of homozygously p53-deficient mice which are prone to develop malignancies, particularly lymphomas and haemangiosarcomas, with a high incidence soon after birth [24,25].

These findings prompted us to examine twelve lymphoma patients seen at the University of Freiburg Medical Center who had a positive family history of malignant lymphoma and/or a diagnosis of metachronous lymphoma for the presence of p53 germline mutations within exons 4–8, where the large majority of p53 mutations have been described.

PATIENTS, MATERIALS, AND METHODS

Between 1991 and 1993, familial and intraindividual clustering of lymphomas was observed in twelve patients treated and followed up for malignant lymphoma at the University of Freiburg Medical Center. Histological diagnoses were made at the Institute of Pathology, University of Freiburg. After obtaining informed written consent, patients with HD were treated according to the protocols of the German Hodgkin's Lymphoma Study Group. The following criteria were applied for familial aggregation of lymphoma: histologically confirmed diagnosis of HD or NHL in the index patient, and malignant lymphoma in at least one first-degree relative. Metachronous lymphoma was defined as follows: in HD patients with at least one second malignant neoplasm (SMN) that was histologically confirmed as NHL occurring at least 6 months after initial diagnosis of HD; in NHL patients with at least one SMN of HD, or of NHL with a histological subtype different from the initial NHL, occurring at least 6 months after diagnosis of the preceding, treated lymphoma. Personal and family history of malignancy was obtained by means of a patient interview and review of medical records.

Cells, Cell Lines, DNA Preparation

Heparinized venous blood was collected from consenting patients and, where available, first-degree relatives, and subjected to density separation by Ficoll gradient. DNA was isolated from mononuclear cells by ultracentrifugation over a 5.4 M CsCl gradient after lysis of cells in guanidinium isothiocyanate [26], or nuclei were obtained by hypotonic cell lysis and further purified by proteinase K digestion and ammonium acetate precipitation as described [27].

The following cell lines were used as positive controls for p53 mutations: U-266 (myeloma, codon 161, exon 5), Jurkat (T-lymphoblasts, codon 196, exon 6), CEM (T-lymphoid, codon 175, exon 5, codon 248, exon 7), HEK 1B (endometrial carcinoma, codon 248, exon 7), BT 474 (codon 285, exon 8), and A 431 (epithelial carcinoma, exon 8), which were kindly provided by Carl Miller, Ph.D., Cedars-Sinai Medical Center/UCLA School of Medicine, Los Angeles, California and Drs. Kiechle and Ikenberg, Dept. of Gynecology, University of Freiburg, Freiburg, Germany, respectively. Eight DNA samples from patients with LFS and different known germline p53 mutations were kindly provided by Drs. Fred Li and Sigitas Verselis, Dana-Farber Cancer Institute, Boston, Massachusetts.

PCR and SSCP Analyses

Single-stranded conformation polymorphism (SSCP) analysis was performed as described [28,29]. DNA was subjected to polymerase chain reaction (PCR) in a volume of 20 μl containing 0.1 μg of DNA, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ for exons 4, 5, and 7, 0.7 mM $MgCl_2$ for exon 6, and 1.0 mM $MgCl_2$ for exon 8, 2 mM dATP, dTTP, dGTP, and dCTP (Perkin–Elmer, Langen, Germany; 5 μCi ^{32}P-dCTP (Amersham, Braunschweig, Germany), 10 pmol of each oligonucleotide primer, and 1 unit AmpliTaq DNA Polymerase (Perkin–Elmer). For exon 4, the following primers were used: PX4LT: 5′-GGAATT CAC CCA TCT ACA GTCC-3′ and PX4RT-2: 5′-CTC AGG GCA ACT GAC CGTG-3′ [29]. The primers specific for exons 5–8 were those used by Paquette et al. [30]: exon 5: 5′-TCT GTT CAC TTG TGC CCT GAC TTT C-3′ and 5′-ACC CTG GGC AAC CAG CCC TGT CGT C-3′, exon 6: 5′-CAG GGC TGG TTG CCC AGG GTC CCC A-3′ and 5′-ACT GAC AAC CAC CCT TAA CCC CTC C-3′, exon 7: 5′-GGT CTC CCC AAG GCG CAC TGG CCT C-3′ and 5′-GAG GCT GGG GCA CAG CAG GCC AGT G-3′, exon 8: 5′-TAG GAC CTG ATT TCC TTA CTG CCT C-3′ and 5′-AAC TGC ACC CTT GGT CTC CTC CAC C-3′. PCR was performed for 30 to 36 cycles with a denaturing temperature of 94°C for 1 min (first cycle: 7 min), an annealing temperature of 55°C for exons 4, 5, and 6, 58°C for exon 7 and 60°C for exon 8 for 1 min and elongation at 72°C for 1 min. After amplification, radiolabeled PCR samples were subjected to SSCP analysis: an aliquot of the PCR reaction was diluted 10-fold with loading buffer containing 96% formamide, 20 mM ethylenediaminetetraacetic acid, 0.05% xylene cyanole, and 0.05% bromophenol blue, heated to 95°C for 10 min and then chilled on ice until loading. By use of various DNA samples with known point mutations, the optimal elec-

trophoresis conditions for visualization of mutations were determined by using different glycerol concentrations (0 to 10%) and temperatures (at room temperature, at 4°C) as follows: gels contained 6% polyacrylamide (Sigma, St. Louis, MO), 0.5 × Tris-borate-EDTA and 5% glycerol and were run at 300 V at room temperature for 14 to 18 hr, dried and autoradiographed for 8 to 20 hr at −80°C.

Subcloning and Sequencing of PCR Products

p53 exons 6 and 8 were further characterized by DNA sequencing: the PCR product of a nonradioactive PCR was subcloned and INVaF′ competent bacteria transformed with the pCRTM2.1 vector by using the "TA cloning" kit (Invitrogen, San Diego, CA). After DNA extraction, sequencing of the double-stranded plasmid was performed by using a modified DNA polymerase (Sequenase United States Biochemical, Cleveland, OH, USA). Briefly, DNA was annealed to 4 pmol of the 5′ or 3′ PCR primer of the respective exon in the presence of 1μl 0.1 M DTT, 5 μCi ^{33}P dATP (Amersham, Braunschweig, Germany). After termination of each sequencing reaction and denaturing by boiling for 5 min, half the reaction volume was loaded onto denaturing 6% polyacrylamide/urea gels and electrophoresed at 50°C. Gels were dried and exposed to Kodak X-AR 5 films at room temperature for 12–48 hr. Sequencing of exon 4 was performed with an ABI 373a automated sequencer according to instructions of the manufacturer after purification of the PCR product with Qiaquick PCR purification Kit (Qiagen GmbH, Hilden, Germany).

TABLE I. Patients With Familial and Intraindividual Clustering of Malignant Lymphoma*

Patient	Sex	Age	Histology	Stage	Family history (age at diagnosis)	Treatment
1	M	37	HD-LP	I A	**Sister: ALL (17)** **Brother: HD (29)** Father: LC (49) Mother: BC (46)	COPP/ABVD (4×) RT (mediastinum)
2	M	38	HD-LP	III A	**Father: HD (49)** Mother: BC (58)	COPP/ABVD (4×) RT (total nodal)
3	F	20	HD-NS	II A	**Mother: HD (40)**	COPP/ABVD (2×) RT (extended field)
4	F	40	HD-NS	III B	Mother of pat. 3	COPP/ABVD (3×) CEVD (2×) RT (total nodal)
5	M	60	HD-MC	I A	**Sister: HD-MC (59)**	RT (epipharynx, mantle field)
		60	T-NHL-LB	I A	Father: LC (57)	VACOP-B (6×)
6	M	50	NHL-CB	III B	**Son: HD (24)** **Brother: HD-LP (42)**	VACOP-B (6×) VIPE (1×) BEAM + SCT
7	M	48	CLL	II	**Brother: CLL (51)**	Chlorambucil Prednisone Cyclophosphamide
8	M	2	NHL-CB	II B	**Brother: B-NHL (4)**	Multiple polychemotherapies SCT
9	M	53	T-NHL	I A	Mother: cervical cancer (39) Father: thyroid cancer (81)	IFN-α Chlorambucil
		55	HD-LP	III B	Sister: B.C. (51)	COPP/ABVD (4×) VIPE (1×) BuCy + SCT
10	M	19	HD-NS	III A	No malignancies	COPP/ABVD (2×) RT (mediastinum)
		20	NHL-CB	IV B		VACOP-B (7×) RT (infradiaphragmal)
11	F	30	HD-NS	II A	Mother: colon-ca (66)	RT (supra-/infradiaphragmal) High-dose dexamethasone
		51	MM-IgMk	I A		
12	F	59	HD-MC	II B	Father: colon-ca (61)	RT (supra-/infradiaphragmal) CHOP (4×)
		68	NHL-CB	II A		RT (epipharynx)

*Relatives of patients who also suffered from lymphoid malignancies are indicated in bold type. Polychemotherapy protocols were performed as described [38]. Abbreviations: HD, Hodgkin's disease; LP, lymphocyte predominance; NS, nodular sclerosis; MC, mixed cellularity; NHL, non-Hodgkin's lymphoma; CB, centroblastic; LB, lymphoblastic; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia; BC, breast cancer; LC, lung cancer; RT, radiation therapy; SCT, hematopoietic stem cell transplantation.

RESULTS

Patient Characteristics

Between 1991 and 1993, 12 patients with a familial and/or intraindividual aggregation of malignant lymphoma were seen at the University of Freiburg Medical Center either during initial treatment or during follow-up. The median age of the patients at initial diagnosis of lymphoma was 37 years (range, 2–60 years). Five patients suffered from HD and had a family history of HD or other lymphoma; in patient 1, a striking accumulation of malignancies including HD and acute leukemia in four first-degree relatives was notable, but did not fulfill the rigorous criteria of LFS. Three patients had NHL and a positive family history of lymphoma (Table I). Four HD patients were subsequently diagnosed with metachronous NHL as a SMN. The median time interval between diagnosis of initial and metachronous lymphoma was 12 months (range, 6 months through 9 years). Two patients with lymphoma as SMN had been treated with radiation therapy alone, two others with radiochemotherapy.

p53 Analysis in Patients With Familial and Intraindividual Clustering of Lymphoma

Genomic DNA was isolated from peripheral blood mononuclear cells while patients were in remission and analyzed for the presence of p53 germline mutations in exons 4–8. SSCP analysis of these exons enabled us to detect previously described mutations in 6/6 cell lines (see Patients, Materials, and Methods) and 8/8 patients with known LFS and p53 alterations kindly provided by Dr. Fred Li, Boston (data not shown). Upon SSCP analysis of the p53 coding region in the index patients, a band shift was noted for exon 4 in patient 3 (Fig. 1A, lane 3). Sequencing of subcloned PCR product revealed a known DNA polymorphism in codon 72 (CGC to CCC) causing a substitution of arginine by proline. All other samples analyzed disclosed a band pattern identical to the normal controls. This is also shown for exon 5 (Fig. 1B). To rule out nucleotide substitutions not influencing strand mobility, PCR products of exons 6 and 8 from patients 1, 2, 3, and 5 were also subcloned and sequenced. However, only wild-type sequences were detected (data not shown).

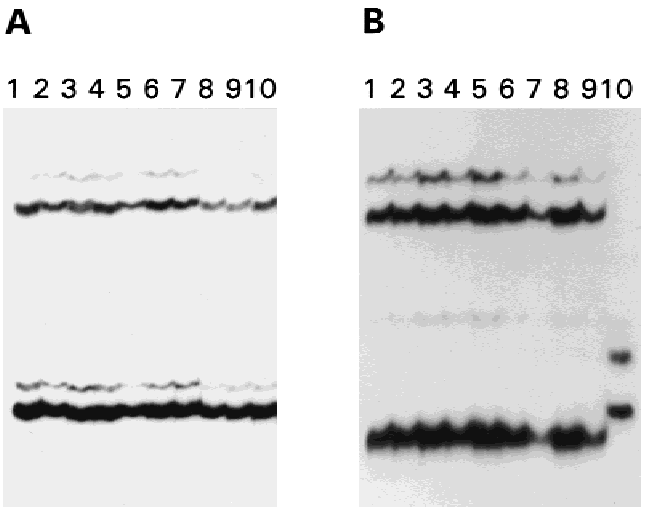

Fig. 1. p53 analysis of patients with familial or intraindividual aggregations of malignant lymphoma. DNA from peripheral blood lymphocytes was subjected to PCR and SSPC as described in Patients, Materials, and Methods. (A) SSCP analysis of p53 exon 4 was performed on DNA from patients 1–6 (lanes 1–6) and patients 8–10 (lanes 8–10). In lane 7, DNA from a patient with multiple myeloma but without metachronous lymphoma or a first-degree relative with lymphoma was analyzed. The gel was run without glycerol at 4°C at 300 V. Note duplex band in lane 3 indicating p53 codon 72 RFLP (confirmed by DNA sequencing). (B) SSCP analysis of p53 exon 5 was performed on DNA from patients 1–6 (lanes 1–6), 8 (lane 7), 10 (lane 8), and 10 (lane 9). As positive control for SSCP, U-266 with known mutation at codon 161 was run in lane 10. The gel was run without glycerol at room temperature at 270 V.

DISCUSSION

Several lines of evidence point to a role of p53 in lymphomagenesis: somatic p53 mutations occur in patients with NHL or HD [17–23], and development of lymphoma is observed at a high rate in several mouse models of p53 alteration, i.e., overexpression of a p53 gene altered by a point mutation [31], and p53 gene deletions [24,25], respectively. Furthermore, NHL and leukemias have occasionally been described as part of the LFS [11,12,32,33] in which germline mutations of p53 are the inherited genetic alteration. In order to determine whether p53 germline mutations occur in familial aggregations of lymphoma, i.e., outside the LFS, we examined patients with a positive family history for malignant lymphoma and/or with metachronous lymphoma. DNA from 12 patients fulfilling the criteria described in Patients, Materials, and Methods was analyzed for the presence of constitutional p53 mutations of exons 4–8, which span the highly conserved p53 gene region, where the large majority of mutations has been detected [33]. However, in our study this region did not reveal any sequence alterations such as those commonly associated with LFS. During completion of the present analysis, two other studies have recently described absence of germline p53 mutations in HD and NHL pedigrees [34,35]. Constitutional p53 mutations within this region, therefore, seem to be a rare event in familial clustering of HD and NHL.

By the assay used, we cannot rule out that p53 inactivation might still be involved in the predisposition to these hematologic malignancies, but the mutation site was situated outside the protein coding region of p53, or the function of the p53 protein could be altered by other

mechanisms. Of note, even in LFS pedigrees the incidence of mutations in the p53 protein coding region is only about 50% [33]. Barnes et al. described a LFS pedigree in which no mutation in the p53 gene could be detected but expression of p53 was abnormally high [36]. Somatic alterations within the 5′ flanking region of the p53 gene have also been described [17,20,27].

The significantly increased risk for secondary malignancies including NHL and HD following radiochemotherapy is well documented and under active investigation. In two of the patients studied by us (patients 11 and 12) the time intervals between diagnosis of the metachronous lymphomas, and the localization of secondary lymphoma in patient 11 within the irradiated area are compatible with a SMN induced by the preceding therapies. However, because only a subgroup of treated lymphoma patients develops SMN, a genetic predisposition has been discussed also for these patients (8,37).

In summary, somatic p53 mutations can clearly contribute to progression of malignant lymphoma, and data derived from murine models suggest a role for constitutional alteration of p53 in lymphomagenesis, which is strengthened by the occurrence of NHL within LFS pedigrees. However, in familial aggregation of lymphoma and metachronous lymphoma occurring outside the spectrum of LFS, altered p53 is unlikely to contribute to predisposition to these disorders, and studies identifying other tumor suppressor gene(s) involved are warranted.

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